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Rotavirus VP7-gene selection during coinfections in CaCo-2 cells

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ABSTRACT

A previous rotavirus epidemiological survey in Córdoba, Argentina, revealed an unusually high frequency of mixed G-type infections (41.5%). The genotype distribution of those mixed infections showed that the most prevalent G-type combinations were G1 + G4 (65.0%), G1 + G2 (12.5%), G2 + G4 (3.1%) and G1 + G9 (2.5%). In the present study we analyzed the competitive growth in CaCo-2 cell cultures of strains from the most frequent rotavirus G-type coinfections in order to explain some aspect of the dynamic of G-type replacement along the time. Our results indicated that G1-type was preferentially selected compared with G2 and G9-genotypes, meanwhile, G1–G4 coinfections showed an efficient co-amplification of both types. Interestingly, this mirrored the high detection rates of both genotypes as single and mixed infections (G1 + G4, 65.0%) in our region. On the other hand, G2-type revealed a better amplification rate with respect to G4-type. Fluctuant rates in the prevalence of different genotypes usually observed along the time could, in part, be explained by successive replacement of strains with different growth characteristics. We hypothesized that one aspect of these different fitnesses can be measured as differential growth in culture of the strains contained in the sample of a mixed infection. Our findings here provide the first data supporting the validity of the competitive replication in vitro to better understand rotavirus G-type circulation patterns.

1. Introduction

Group A rotaviruses are the single most important etiological agent associated with gastroenteritis in infants and young children (Albert and Robert, 1996; Iturriza-Gómara et al., 2000). It is estimated that each year 440,000 children under five years of age die of rotavirus gastroenteritis, two million are hospitalized and 25 million require an outpatient visit (Parashar et al., 2003). In poor countries, approximately one child in every 250 will die of rotavirus disease by five years of age (Parashar et al., 2003).

Rotaviruses contain 11 segments of double-stranded RNA within a core shell and are members of the *Reoviridae* family. Rotaviruses outer capsid proteins, VP7 (glycoprotein) and VP4 (spike protein), independently induce neutralizing antibodies (Greenberg et al., 1983; Hoshino et al., 1985; Offit and Blavat, 1986). Based on these proteins, a dual classification system of group A rotaviruses has been introduced (Estes, 2001): G-types (derived from VP7 protein) and P-types (derived from VP4 protein). At least 19 G-genotypes have been recognized by neutralization assay and 27 P-genotypes have been identified by hybridization or sequence analysis (Khamrin et al., 2007a; Matthijnssens et al., 2008). The major human G-types are G1, G2, G3, G4 and G9, which, combined with the P-types P[8], P[4] and P[6], account for more than 80% of rotavirus-associated gastroenteritis episodes worldwide (Gentsch et al., 2005; Santos and Hoshino, 2005a).

During the past two decades, different organizations including the World Health Organization (WHO), the Institute of Medicine and the Global Alliance for Vaccines and Immunization (GAVI), have identified rotavirus vaccines as a priority for development (WHO, 2000). This decision has been based primarily on the enormous toll of rotavirus disease. Since the year 2006 two new rotavirus vaccines are commercially available in Argentina, a monovalent live attenuated vaccine containing a P1A[8]G1 strain (Rotarix; GlaxoSmithKline Biologicals) and a pentavalent bovine-human G1 to G4, P[8] reassortant vaccine (Rotateq: Merck and Sanofi Pasteur MSD). Although large-scale safety and efficacy studies of both rotavirus vaccines have shown excellent efficacy against severe rotavirus gastroenteritis (Ruiz-Palacios et al., 2006; Matson, 2006), the lack of clear data about the heterotypic protection underline the importance of virological surveillance,

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rotavirus strain characterization (Gentsch et al., 2005; Perez-Schael et al., 1990; Velázquez et al., 1996) and the impact of these vaccines in diminishing the diarrhea illness.

In a previous study undertaken in Córdoba, Argentina (Barril et al., 2006) the epidemiological pattern of group A rotaviruses circulating during the period 1979–2003 was evaluated. This study showed a rotavirus G-type circulation pattern that was defined by local characteristics and others shared with most of the world's regions. The local characteristics involved a high frequency of mixed G-type rotavirus infections (44.6%) and the circulation of the emerging G9-genotype since 1980.

With the aim of getting some insight on the dynamic of rotavirus strains circulation in our region, the genotype distribution of the coinfections was analyzed and, in addition, an in vitro system was assayed inoculating CaCo-2 cell cultures with samples from most of the relevant mixed infections observed in the population. We studied whether selective advantages for certain rotavirus genotypes exist during cells passage after coinfections, and whether that genotype can therefore be preferentially selected in virus populations.

2. Materials and methods

2.1. Rotavirus mixed G-type analysis

2.1.1. Heminested-PCR with individual type-specific primers

The amplicons of the full length VP7-gene obtained by RT-PCR from fecal samples characterized as mixed G-type by multiplex PCR infections were confirmed for each genotype involved in the coinfection by individual PCR reactions using the degenerate primer End9 and the type-specific primer.

2.1.2. Sequencing

Thirty samples were chosen to verify the authenticity of the resulting DNA amplicons after G-typing. The heminested-PCR amplicons were purified and sequenced in both directions (with the appropriate primers) using the dideoxy-nucleotide chain terminator method with Big Dye TM terminator Cycling Conditions on an automated sequencer (3730xl). Sequences were assembled and analyzed with the BioEdit version 7.0.5.2 alignment editor and were compared against the GenBank database using BLAST program.

2.2. Viral progeny during coinfections in CaCo-2 cells

2.2.1. Culture of G-type rotavirus coinfections

Stool samples obtained from children naturally coinfected with two strains of different VP7-genotypes were inoculated onto CaCo-2 (human colon adenocarcinoma) cells. Due to the coinfection nature of the samples, the inoculums corresponding to each G-type could not be quantified. Therefore, the criterion for inclusion of the samples was that they should have equivalent amounts of PCR products for each genotype involved in the coinfection.

CaCo-2 cells were coinfected with pairs of human rotavirus genotypes and passaged three times employing the culture fluid as inoculums for the following passage. The four most prevalent kinds of coinfections were assayed in cell cultures: (A) G1 and G4; (B) G1 and G2; (C) G2 and G4; and (D) G1 and G9. Four samples of each kind of coinfection were individually analyzed and each sample was inoculated by sextuplicate, therefore a 24-well plaque was used for each kind of coinfection. Sextuplicate cultures were independently analyzed in order to determine the reproducibility for each set of viruses.

2.2.2. CaCo-2 cell line infection

The 16 stool samples selected for this study were clarified, treated with chlorophorm, antibiotics (penicillin/streptomycin) and fungizone. An aliquote of 10% fecal suspension was pretreated with an equal volume of trypsin (Difco 1:250, 20 μg/ml) and incubated at 37 °C for 30 min. Then, 100 μl of each preactivated fecal supernatant was inoculated by sextuplicate in a 24-well microculture plate onto a confluent monolayer of CaCo-2 cell line (O'Neill et al., 1996). After a 60 min adsorption at 37 °C with agitation, the extracts were removed. The cultures were maintained in Eagle's minimum essential medium free of fetal bovine serum (FBS), supplemented with L-glutamine, non essential aminoacids, sodium piruvate and tripsin (5 μg/ml); and incubated at 37 °C with 5% CO_2 (Cumino et al., 1998).

2.2.3. Culture harvest

Plates were checked daily for the presence of cytopathic effect (CPE), and passaged two times at intervals between 5–7 days irrespective of the presence or absence of CPE. Finally, the cultures were harvested and submitted to three freeze-thaw cycles.

2.2.4. Viral dsRNA extraction

Viral dsRNA was extracted from culture supernatants by the phenol–chlorophorm method followed by alcohol precipitation according to standard procedures (Perry et al., 1972). After drying, the pellets were diluted in 20 μl of sterile distilled water.

2.2.5. RT-PCR (Gouvea et al., 1990)

Extracted RNA was reverse-transcribed into gene 9 (VP7) full length cDNA with the generic primers Beg9/End9. Then, the cDNA product was used as template for PCR VP7-amplification with the same Beg9/End9 pair of primers.

2.2.6. Genotyping

The VP7 full length PCR products were used as templates in combination with two cocktails of type-specific forward primers and the generic reverse primer End9 for G-genotyping (Gouvea et al., 1990). The cocktails were as follows: G1 (aBT1), G2 (aCT2), and G3 (aET3) in one mixture, and G4 (aDT4), G8 (aAT8) and G9 (aFT9) in the second one. The amplicons were analyzed by electrophoresis on 10% polyacrylamide gels (Laemmli, 1970) and visualized after silver staining as described elsewhere (Herring et al., 1982).

3. Results

3.1. Global analysis of mixed G-type rotavirus infections

Out of the 172 mixed G-type infection samples detected during a period of 25 years in Córdoba city, Argentina, 12 could not be confirmed by PCR using individual specific primers followed by sequence analysis: 11 samples could not be re-amplified as mixed infections and one sample could not be confirmed by sequence analysis. Therefore, the total number of mixed G-type infections slightly dropped from 172 (44.6%) to 160 (41.5%) and the most prevalent mixed G-type double infections were G1 + G4 (104/160, 65.0%), G1 + G2 (20/160, 12.5%), G2 + G4 (5/160, 3.1%) and G1 + G9 (4/160, 2.5%). Table 1 depicts mixed infection rates and G-type distribution during the studied period. The proportional distribution of G-types in rotavirus mixed infections was G1, 43.6%; G2, 12.0%; G3, 2.0%; G4, 38.6%; G8, 0.3%; and G9, 3.5%.

3.2. G-types fitness during rotavirus coinfections in CaCo-2 cells

In order to roughly estimate the relative amount of VP7-gene yielded after viral competition in cell cultures, supernatants from infected cultures were analyzed by RT-PCR and the products were visualized by PAGE/SS. Table 2 shows the frequency of G-type detection rate by sample analyzed by sextuplicate at the 1st, 2nd